Color SARS-CoV-2 RT-LAMP Diagnostic Assay

Version 2.0 – Updated 10.14.20

Executive Summary

The Color SARS-CoV-2 RT-LAMP Diagnostic Assay utilizes real time reverse transcription loop-mediated isothermal amplification technology to simply and efficiently detect the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The assay procedure consists of collection of patient respiratory specimens, RNA extraction, reverse transcription, and loop-mediated isothermal amplification. Results are interpreted by colorimetric read-out. The Color assay was demonstrated to have a limit of detection of 0.75 copies of viral RNA per µl of primary sample and showed 100% positive and negative agreement for 539 patient samples with previous results from another laboratory.

Introduction

Broad access to diagnostic testing is a crucial component in controlling the spread of coronavirus disease 2019 (COVID-19). In response to the ongoing pandemic, Color developed a scalable diagnostic test for the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the RNA virus that causes COVID-19. The Color assay was designed to be high throughput, cost-efficient, and highly automated. Furthermore, the assay utilizes processes that are orthogonal to other SARS-CoV-2 diagnostic tests in order to reduce strain on reagent supply chains.

The Color SARS-CoV-2 RT-LAMP Diagnostic Assay is used for *in vitro* qualitative detection of SARS-CoV-2 in respiratory specimens from patients as recommended for testing by public health authority guidelines. The test first received Emergency Use Authorization (EUA)[1] from the United States Food and Drug Administration (US FDA) on May 18, 2020,[2] and is performed in the Color CLIA certified high-complexity laboratory (Burlingame, CA). Color's assay utilizes colorimetric real time reverse transcription loop-mediated isothermal amplification (RT-LAMP) technology to detect SARS-CoV-2.

Loop-mediated Isothermal Amplification (RT-LAMP)

RT-LAMP is similar to other nucleic acid amplification diagnostic tests (e.g., real time reverse transcription polymerase chain reaction (RT-PCR)) that detect the presence of RNA, but there are a few key differences. Unlike RT-PCR, RT-LAMP occurs at a single temperature and thus does not require sophisticated thermocycling instrumentation. Instead, isothermal amplification is carried out through a set of primers that create loop-structures for self-priming exponential amplification. These primer sets consist of six primers designed to target a specific gene. Instead of using high temperature to denature the double-stranded DNA, the DNA polymerase has a strand displacement activity. Together, the primer sets generate amplification products that consist of inverted repeats of the target sequence on the same strand, which in turn create self-priming loop structures. The amplification can then be carried out rapidly at a single temperature, generating up to 10^9 copies of the target in less than an hour.[3]

Test principle

The Color assay includes a reverse transcription step followed by colorimetric LAMP. The assay uses two SARS-CoV-2 specific primer sets. In one version of the assay the two viral primer sets target the SARS-CoV-2 nucleocapsid gene (N) and the envelope gene (E), and in the other version of the assay they target the spike gene (S) and ORF1a region. A third primer set that targets the human ribonuclease P (RNaseP) transcript is used as a positive human control in both assay versions.

The Color assay uses RT-LAMP chemistry modified from New England BioLabs (NEB, E1700). Nucleotid incorporation by the DNA polymerase during amplification releases protons, changing the color of a pH sensitive dye. Reaction performance is measured by the change in the ratio of light absorbed at 430 and 560 nm. Each of the three primer set reactions are monitored at 60 second intervals (Figure 1).

Quantification of absorbance change is performed by determining the reaction's absorbance ratio at baseline, the amplification slope, and the ratio gain between baseline and endpoint.

Acceptable sample types

The Color assay is intended for the qualitative detection of nucleic acid from SARS-CoV-2 in nasopharyngeal (NP) swabs, oropharyngeal (OP) swabs, anterior nares (AN) swabs, mid-turbinate nasal (MTN) swabs, NP wash/aspirate or nasal aspirates, and bronchoalveolar lavage specimens collected from individuals suspected of having COVID-19 by a healthcare provider. Respiratory specimens should be collected and transported in appropriate transport media such as research use only DNA/RNA Shield™ media (Zymo Research, R1100-250), viral transport media, universal transport media, or saline. The following swabs were used during validation of the assay: NP and OP swabs (Copan), PurFlock Ultra (Puritan, 25-3206-U), and disposable sampling swab (Jiangsu Hanheng Medical Technology Co., Ltd, A-01).

Dry AN swabs have been validated for use with the Color Assay. A validation was performed with a set of 35 positive and 2,117 negative samples. Results from swab samples collected and transported dry in no stabilization media were compared to swabs collected and transported wet in DNA/RNA Shield media. The positive predictive agreement (PPA) was 94.3% and the negative predictive agreement (NPA) was 99.9%.

Methods

Sample preparation and RNA extraction

The Color SARS-CoV-2 RT-LAMP Diagnostic Assay methodology consists of the following steps. First, samples from respiratory specimens are collected and transported dry or in appropriate transport media. Samples are then transferred to a 96-well plate for bead-based RNA extraction using Hamilton STARlet automated instrumentation. RNA is extracted using the Viral DNA/RNA 300 Kit H96 (Perkin Elmer, CMG-1033) and automated on the Perkin Elmer Chemagic360 instrument platform. Extracted RNA is then transferred from the extraction elution plate to a 384-well plate using Hamilton STARlet automated instrumentation. Automated RT-LAMP reaction setup is performed using Hamilton STAR instrumentation.

Table 1. Primer sequences used in the Color SARS-CoV-2 RT-LAMP Diagnostic Assay

Primer Set	Primer ID	Sequence
N-gene (SARS-CoV-2, nucleocapsid)	F3	AACACAAGCTTTCGGCAG
	B3	GAAATTTGGATCTTTGTCATCC
	FIP	TGCGGCCAATGTTTGTAATCAGC-CAAGGAAATTTTGGGGAC
	BIP	CGCATTGGCATGGAAGTCACTTT-GATGGCACCTGTGTAG
	LF	TTCCTTGTCTGATTAGTTC
	LB	ACCTTCGGGAACGTGGTT
E-gene (SARS-CoV-2, envelope)	F3	CCGACGACGACTACTAGC
	B3	AGAGTAAACGTAAAAAGAAGGTT
	FIP	ACCTGTCTCTTCCGAAAC-GAATTTGTAAGCACAAGCTGATG
	BIP	CTAGCCATCCTTACTGCGCTACT-CACGTTAACAATATTGCA
	LF	TCGATTGTGTGCGTACTGC
	LB	TGAGTACATAAGTTCGTAC
ORF1a (SARS-CoV-2, open reading frame 1a)	F3	CGGTGGACAAATTGTCAC
	B3	CTTCTCTGGATTTAACACACTT
	FIP	TTACAAGCTTAAAGAATGTCTGAA-CACT
	BIP	TTGAATTTAGGTGAAACATTTGT-CACG
	LF	TCAGCACACAAAGC-CAAAAATTTATTTTTCTGTG-CAAAGGAAATTAAGGAG
	LB	TATTGGTGGAGCTAAACTTAAAG-CCTTTTCTGTACAATCCCTTTGAGTG
S-gene (SARS-CoV-2, spike)	F3	TCTATTGCCATACCCACAA
	B3	GGTGTTTTGTAAATTTGTTTGAC
	FIP	CATTCAGTTGAATCACCACAAAT-GTGTGTTACCACAGAAATTCTACC
	BIP	GTTGCAATATGGCAGTTTTTGTA-CATTGGGTGTTTTTGTCTTGTT
	LF	ACTGATGTCTTGGTCATAGACACT
	LB	TAAACCGTGCTTTAACTGGAATAGC
RNaseP (human, ribonuclease P)	F3	TTGATGAGCTGGAGCCA
	B3	CACCCTCAATGCAGAGTC
	FIP	GTGTGACCCTGAAGACTCG-GTTTTAGCCACTGACTCGGATC
	BIP	CCTCCGTGATATGGCTCTTC-GTTTTTTTCTTACATGGCTCTGGTC
	LF	ATGTGGATGGCTGAGTTGTT
	LB	CATGCTGAGTACTGGACCTC

RT-LAMP

Incubation and data collection for the Color assay is performed on the Biotek NEO2 microplate reader. The plate is incubated at 65°C for 70 minutes. During this isothermal reaction, reverse transcription and loop-mediated isothermal amplification occur. Extracted RNA is processed through a colorimetric RT-LAMP procedure[4] using two primer sets targeting the SARS-CoV-2 (either the N-gene[5] and the E-gene[5] or the S-gene and ORF1a respectively), and a third control primer set targeting human RNaseP[7] (Table 1). The reaction color change initiated by amplification is measured spectrophotometrically by taking the absorbance at 430 and 560 nm over the 70 minutes using the Biotek NEO microplate reader. Reactions displaying a color shift indicate that the target sequence is present.

Control Materials

Two control materials and one human control primer set are included as part of the Color assay. The expected results for each primer set for these control materials are described in Table 2.

Two "extraction controls" are included in each extraction batch and carried through the full end-to-end process. The first extraction control is a positive control consisting of DNA/RNA Shield™ media spiked with extracted human total nucleic acid and synthetic viral SARS-CoV-2 RNA (Twist, Synthetic SARS-CoV-2 RNA Control 1 (MT007544.1)) at 5x the limit of detection (LoD). A lack of amplification in this positive control would indicate that there is a reagent or process failure during extraction or RT-LAMP. The second extraction control is a no template control (NTC) consisting of DNA/RNA Shield™ media alone. Amplification in the NTC would indicate that there was contamination of extraction and/or RT-LAMP reagents.

Additionally, each clinical sample is processed through RT-LAMP with a control primer set that targets human RNaseP (this is in addition to the two primer sets targeting the SARS-CoV-2). Lack of sample amplification with the RNaseP primer set would indicate extraction failure for that sample.

Table 2. Expected results of batch controls

	Expected signal	
Control	**SARS-CoV-2 targets**	**Human RNaseP**
Extraction positive	Both positive	Positive
Extraction NTC	Both negative	Negative

NTC, no template control.

Data interpretation

Interpretation procedure

Visible light absorbance (A) in each well is measured once per minute, from time (t) = 0 minutes to t = 70 minutes, and the absorbance ratio (A430/A560) at each point is calculated (Figure 1). Three points are identified: the absorbance ratio at baseline, the absorbance ratio at the endpoint, and the maximum rate of amplification.

- **Absorbance ratio at baseline:** The derivative of the absorbance ratio is calculated, and this curve is smoothed using a rolling average of nine adjacent data points. The baseline time point is identified as the first point at which the curve flattens out after the initial color change and the slope drops below 0.005. This point must fall between 5-25 minutes with absorbance ratios between 1.2 - 1.6. If it does not meet these parameters, the baseline assessment is set to "failed". The baseline time point is used to calculate the **baseline ratio**, which is the average of five adjacent data points.
- **Absorbance ratio at the endpoint:** For the endpoint, set at 55 minutes, the absorbance ratio is quantified using a rolling average of five adjacent data points. The **ratio gain** is defined as the difference between the absorbance ratios of the end point and baseline point.
- **Maximum amplification rate:** The maximum amplification rate is calculated as the maximum slope achieved between 20 minutes and the endpoint, using a rolling average.

The ratio gain and the maximum amplification rate are used in the interpretation for each primer set, as outlined in Table 3.

Possible Clinical Results

Using the established interpretation procedure, a result for the qualitative detection of SARS-CoV-2 can be assigned. First, the expected results per primer for the four control materials run with each batch must be observed as listed in Table 2. If the control results are as expected, then clinical results are interpreted as detailed in Table 4.

Table 3. Ratio gain interpretation for each primer

A430/A560 ratio gain	Maximum amplification rate	Interpretation
≥ 0.25	any	Positive Signal
< 0.25	any	Negative Signal

Figure 1. Representative absorbance data from RT-LAMP positive control

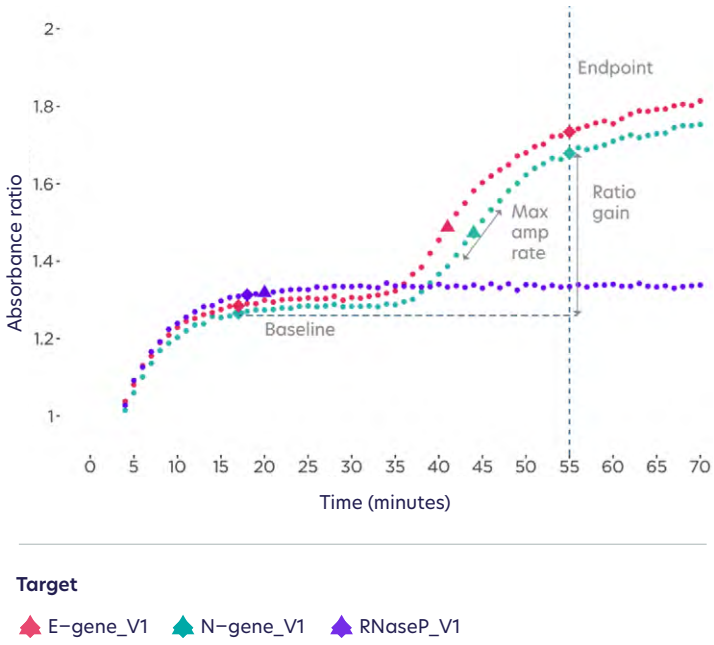

Table 4. Sample results and interpretation

Signal			
SARS-CoV-2 targets **E-gene** **N-gene** **ORF1a**	**Human RNaseP**	**Interpretation**	**Action**
Both SARS-CoV-2 targets +	+	SARS-CoV-2 DETECTED	Report results to physician, patient, and appropriate public health authorities.
One SARS-CoV-2 target +	+	INCONCLUSIVE	Re-extract from residual sample and repeat RT-LAMP. If the repeated result remains inconclusive, report result to physician and appropriate public health authorities. Report indicates that a new sample should be collected.
Both SARS-CoV-2 targets -	+	SARS-CoV-2 NOT DETECTED	Report results to physician, patient, and appropriate public health authorities.
Any combination of + or -	-	FAILED	Re-extract from residual sample and repeat RT-LAMP. If the repeated result remains FAILED, report result to physician and appropriate public health authorities. Report indicates that a new sample should be collected.

Validation

The Color SARS-CoV-2 RT-LAMP Diagnostic Assay was evaluated for analytical sensitivity and clinical performance. This validation data was reviewed by the US FDA, and the assay was granted EUA for the duration of the public health emergency.

Analytical Sensitivity

Limit of Detection

The LoD is defined as the lowest concentration at which 19/20 replicates (or approximately 95% of all true positive replicates) are positively detected. The LoD of the Color SARS-CoV-2 RT-LAMP assay was established using a dilution series of SARS-CoV-2 genomic RNA (ATCC, VR-1986D) spiked into a negative AN clinical matrix in DNA/RNA Shield™ media. Each sample was processed through the entire assay, beginning with RNA extraction. This experiment was done in two phases. The first phase established the preliminary LoD, while the second phase verified the limit.

In phase I, five replicates of six different concentrations ranging from 100 copies per µl to 0.01 copies per µl in the primary sample were processed through the assay. A preliminary LoD was identified as 0.5 copies per µl, which was the lowest concentration of SARS-CoV-2 at which ≥ 95% replicates were detected.

In phase II, the LoD was verified by testing 20 additional individual samples at 0.25 copies per µl, 0.5 copies per µl, 0.75 copies per µl, and 1 copy per µl. All samples were extracted independently. 20/20 replicates were detected at 0.75 copies per µl, therefore 0.75 copies per µl was confirmed as the lowest concentration of SARS-CoV-2 at which ≥ 95% replicates were detected and is the LoD for the Color SARS-CoV-2 RT-LAMP assay.

Additional evaluations

Additional analytical sensitivity evaluations were performed to assess inclusivity, cross-reactivity, and interfering substances. The full results of these evaluations are detailed in the Color SARS-CoV-2 RT-LAMP Diagnostic Assay EUA summary[2] and summarized here.

Inclusivity analysis

The inclusivity analysis was performed by aligning the primer sequences designed for the Color assay against SARS-CoV-2 sequences deposited at Global Initiative on Sharing All Influenza Data (GISAID) on April 2, 2020. This set includes all 2303 complete SARS-CoV-2 sequences that had been annotated as "high coverage." All primer sets in the Color assays have a 100% match with the vast majority of COVID-19 strains: 97.3% for N-gene, 99.3% for E-gene, 97.7% for S-gene, and 98.2% for ORF1a. In all strains with a mismatch in one primer set, the other primer set has a 100% match. Therefore, 100% of the publicly deposited SARS-CoV-2 sequences are considered detectable by this assay.

Cross-reactivity/exclusivity analysis

The cross-reactivity/exclusivity analysis was performed in two phases. In the first phase, *in silico* analysis was performed by aligning the primer sequences designed for the Color assay against the sequences of a total of 19 common viruses and coronaviruses related to SARS-CoV-2. With the exception of SARS-CoV-1, which is closely related to SARS-CoV-2, none of the common viruses have a match against the total sequence length of the SARS-CoV-2 primers greater than the recommended threshold of 80%. Of note, there has not been a case of SARS-CoV-1 since 2004 according to the US Centers for Disease Control and Prevention,[8] rendering the observation of cross-reactivity unlikely. However, the cross-reactivity with SARS-CoV-1 was tested experimentally in the second phase.

For the second phase, the Color assay was used to test cross-reactivity/exclusivity with other organisms. Samples were prepared by spiking (inactivated) purified, intact viral particles, cultured RNA, or bacterial cells into negative buccal swab matrix and processed in triplicate through the end-to-end assay. A total of 51 organisms were evaluated. All results, including SARS-CoV-1, were negative on all three replicates.

Interfering substances

Finally, interfering substances which could be found in respiratory samples endogenously or exogenously were tested to evaluate the extent, if any, of assay inhibition.

A total of 17 substances (such as nasal spray, tobacco, etc.) were used by healthy volunteers, and AN swab samples were immediately collected in triplicate. Contrived positives were created by spiking synthetic SARS-CoV-2 RNA into a clinical matrix at 5x LoD. None of the substances tested were shown to inhibit or interfere with the assay. All contrived positive and negative samples yielded expected results.

Clinical Performance

Clinical performance was assessed in two phases. In phase I, 46 negative and 46 contrived positive samples, spiked with genomic RNA from SARS-CoV-2 (ATCC, VR-1986D) (10 at 1x LoD, 20 at 1.5x LoD, 10 at 13x LoD, and 6 at 133x LoD), were processed through the assay. Results are presented in Table 4.

Table 4. Contrived samples results

		Sample detection rate (n/n)		
		SARS-CoV-2 target		Human target
Concentration of SARS-CoV-2	**Samples (n)**	**N-gene**	**E-gene**	**RNaseP**
0 (negative control)	46	0/46	0/46	0/46
1x LoD (0.75 cp/µl)	10	10/10	10/10	10/10
1.5x LoD (1 cp/µl)	20	20/20	20/20	20/20
13x LoD (10 cp/µl)	10	10/10	10/10	10/10
133x LoD (100 cp/µl)	6	6/6	6/6	6/6

LoD, level of detection. cp, copies.

In phase II, a total of 539 patient samples (37 positive and 502 negative) were processed through the Color assay and compared against external results generated by the Clinical Research Sequencing Platform (CRSP) at the Broad Institute of MIT and Harvard (Cambridge, MA). These samples consisted of 539 NP swabs collected by healthcare providers from patients seeking SARS-CoV-2 testing. These samples had been previously tested at CRSP and contained 37 positive and 502 negative samples. All sample results from the Color assay matched those generated by the CRSP.

Results are shown in Table 5. Positive predictive agreement and negative predictive agreement were determined by comparing Color's observed results with expected results from previous testing at a different laboratory.

Table 5. Clinical sample positive and negative agreement results

		Previous assay result		
		Positive	**Negative**	**Total**
Color assay result	**Positive**	37	0	37
	Negative	0	502	502
	Total	37	502	539
Positive agreement		100% (37/37)		
Negative agreement		100% (502/502)		

For the assay version with the S-gene and ORF1a, a clinical evaluation was performed by analyzing a total of 1370 clinical samples that were processed in parallel through the Color SARS-CoV-2 RT-LAMP Diagnostic Assay using the originally validated E-gene and N-gene primers, as well as the new S-gene and ORF1a primers. All samples in this analysis were consecutive prospectively collected samples being processed for clinical testing and yielded a positive or negative result. Residual samples that were processed with the new primers sets were de-identified, and the operators were blinded to previous results.

PPA and NPA were determined by comparing results obtained using the current group of primers (E- and N-gene) against those obtained using the new primer group (S-gene and ORF1a). Results are shown in Table 6. The positive percent agreement was 100% and negative percent agreement was 99.85%.

Table 6. Clinical evaluation of assay using S-gene and ORF1a

		E-gene and N-gene assay result			
		Positive	**Negative**	**Inconclusive**	**Total**
S-gene and ORF1a assay result	**Positive**	36	0	0	36
	Negative	0	1332	2	1334
	Total	36	1332	2	1370
Positive agreement		100% (36/36)			
Negative agreement		99.9% (1332/1334)			

Conclusion

The Color SARS-CoV-2 RT-LAMP Diagnostic Assay utilizes two SARS-CoV-2-specific primer sets and one human-specific primer set to determine the presence of the virus and the integrity of the sample. The assay has a limit of detection of 0.75 copies per µl of primary sample. Clinical performance evaluations demonstrated that the assay performed as expected over a broad range of viral RNA concentrations and had 100% positive and negative agreement for patient samples with previous results from other laboratories. The assay received EUA from the US FDA in respiratory specimens from individuals suspected of having COVID-19 by a healthcare provider.

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